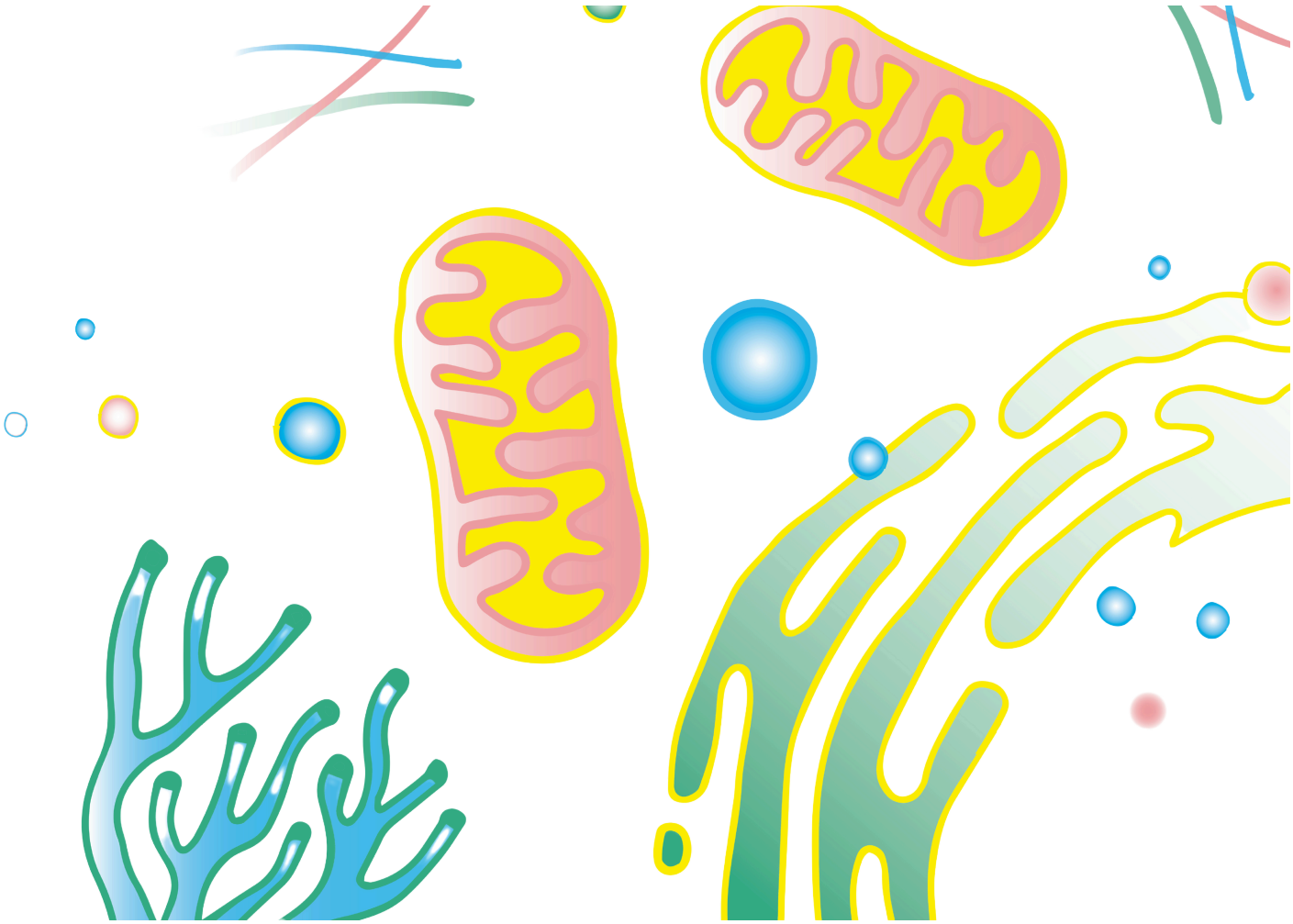
User Protocol

CytoCHECK SPAchip®

OHrad ROS Single-Detection Kit

Product Reference: S-003-ROSG

For use with fluorescence microscopy, cell imaging platforms and flow cytometry.
Research use only.



Arrays for Cell Nanodevices S.L.
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ISO 9001
BUREAU VERITAS Certification

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1. General Introduction

CytoCHECK SPAchip® assay kits are novel cell-based assays for living single-cell developed by A4cell that bring together the fields of nanotechnology and cell biology. CytoCHECK SPAchip® kits are composed of fluorescently labeled silicon microparticles -SPAchips®- that can be internalized in the cytosol of cultured cells to monitor changes in specific intracellular analyte concentrations for long periods of time.

CytoCHECK SPAchip® OHrad ROS Single-Detection Kit allows detecting and measuring hydroxyl radicals by changes in green fluorescence intensity, which allows a more comprehensive study of reactive oxygen species (ROS) in live cell physiology, proliferation, differentiation, cell death, and apoptosis.

CytoCHECK SPAchip® Single-Detection kits are optimized for its use with confocal microscopes and HCS/HCA analyzers with 20X or over magnification objectives, yet widefield fluorescence microscopes and imaging system with fixed wavelength filters can be also used. Flow cytometers have additionally been validated to analyze intra and extracellular SPAchips.

SPAchip® kits can be handled as common fluorophores and chemical probes for cell biology. After resuspending them in the assay buffer (a SPAchip®-to-cell ratio of 3:1 is recommended), an overnight incubation is required to allow SPAchip® to be incorporated in the cytosol[1]. SPAchip® will remain in the cytosol for longer than one month to monitor cell culture progression.

[1] CytoCHECK SPAchip® kits are optimized for its use with adherent cells. Some cells may need a lipofection step to maximize SPAchip internalization. Please contact with A4Cell for further information or questions about your cell model.

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2.Contents and Storage

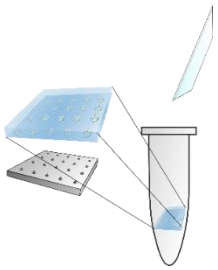
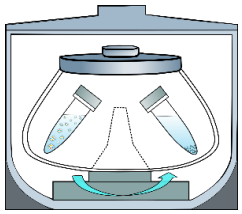
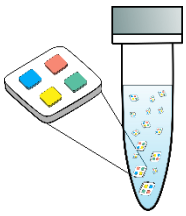
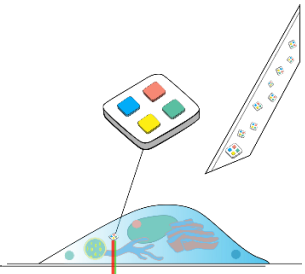
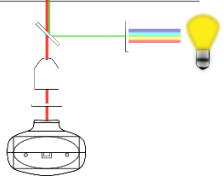
Each CytoCHECK SPAchip® OHrad ROS Single-Detection Kit contains:

Materials		Units
ASSAY SPAchip® tube (embedded in a solid fluorescence-protective soluble film)	~2.5x10^6 ASSAY SPAchips	1
ASSAY buffer tube (sterile, cell culture suitable)	5 mL	1
CONTROL SPAchip® tube (non-fluorescent, ready-to-use)	~2.5x10^6 CONTROL SPAchips/mL	1

Storage Information

Upon receipt, each kit should be stored protected from light at 2-8^{0}C.

CytoCHECK SPAchip® OHrad ROS Single-Detection Kit Workflow

	SPAchip® Assay Workflow	Notes	
ASSAY SPAchip® Preparation	***Dissolve ASSAY SPAchip® tube film***	Thoroughly **dissolve** ASSAY SPAchip® tube solid film in assay buffer (vortexing might be required).	
	Wash with assay buffer (x2)	**Centrifugate** tubes at. 4,300 xg for 10 min. Discard supernatant and keep SPAchip® containing pellet. **Resuspend** the SPAchips in 1mL ASSAY Buffer. **Repeat x2**.	
	Resuspend	**Resuspend** the ASSAY SPAchips in 100 µL ASSAY Buffer to obtain a stock solution of $2.5x10^6$ SPAchips/100µL*.	
	Store	***ASSAY* SPAchip® stock solution** can be **stored** protected from light at 4^0C for up to 6 months.	
Cell Assay	***Cell Culture***	Seed cells (optimization will be required depending on the cell type and assay conditions) in a multi-well plate. Incubate the cells up to 50-70% confluence*.	
	SPAchip dilution	**Dilute** *CONTROL* and *ASSAY SPAchip* stock solution in cell culture medium to obtain a final SPAchip® to cell ratio of 3:1*.	
	Addition to Cell Culture	**Aspire** the cell medium and add SPAchip®-loaded fresh culture medium*.	
	Incubation	**Incubate** overnight in a cell incubator (37^0C, 5% CO_2) to allow SPAchip® internalization by the cells.	
Fluorescence Quantification	***Data Acquisition***	Use **green fluorescence channel** to detect **ROS** green signal (excitation 488 nm, emission 520 nm).	
	Data Analysis	Analyze fluorescence intensity units and normalize the data to control conditions.	

*This cell culture protocol only provides a guideline, and it should be modified depending on specific needs.
Please, read whole protocol before starting.

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3. Materials to be Supplied by the User

- Pipettes and pipette tips (1-10 µL, 2-20 µL, 20-200 µL, 200-1000 µL).
- Multi-channel pipettes and pipette tips (50-300 µL) (not mandatory but desirable).
- Vortex mixer.
- Mini-centrifuge.
- Reagent reservoirs.
- Cell culture conditions:
  - Cell culture facilities.
  - Cell culture plate (multi-well).
  - Cell culture media (phenol red free is highly recommended) according to specific cell line.
- Cell imaging system (HCS/HCS, Confocal microscope, imaging plate reader, etc.) with the appropriate fluorescence excitation and emission wavelength filters. At least 20x magnification objective is required for quantitative analysis.
- Image analysis software (Contact A4cell staff for support).

If qualitative analysis is required, it will be necessary:

- Positive controls (e.g. Tert-Butyl hydroperoxide: TBHP; or Doxorrubicin: DOXO).

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4.Assay Procedure

NOTE: <u>Work under sterile conditions.</u> Protect the tubes from light, specially from UV light.

a. ASSAY SPAchip® Preparation:

1. Add 1 mL of assay buffer to ASSAY SPAchip® tubes and mix until complete solubilization of the membrane (vigorous **vortexing** required).

2. Centrifuge the tubes at approx. 4,300 xg (8,000 RPM in a 6 cm minispin rotor) for 10 minutes.

3. Carefully, aspire and discard supernatant and wash pellets with 1 mL of solubilization medium (**Look out not to aspire the pellet!**). Repeat steps 1 and 2.

4. Carefully, aspire and discard supernatant. Resuspend the pellet in **100 µL of assay buffer** to obtain a SPAchip® stock solution. This results in approximately **2.5x10^6 SPAchips/100µL**[1]. Once prepared, ASSAY SPAchip® tube can be stored at 2-8^{0}C protected from light for up to 6 months.

It is essential to properly dissolve the SPAchip® solid film for the success of the assay.

b. ASSAY PROTOCOL (standard protocol for 96-well plate to work with adherent cells):

NOTE: ASSAY and CONTROL SPAchip® dilutions suggested in this section are suggested for 96-well plates. Optimization might be required for different cell types or multiwell plates.

Goal: To demonstrate intracellular ROS monitorization in adherent cells under different treatment conditions by using SPAchip® nanodevices with fluorescence microscopy techniques.

1. **Seed cells** of interest in a multi-well plate following standard protocols. See an example of plate template in **Figure 1**: control wells are non-fluorescent SPAchips; DOXO and TBHP are two examples of compounds that can be used as positive controls for increasing ROS signal levels. Blank conditions with the sample medium should be used as negative control ; *sample 1* , *2* , etc are the measurements of interest whose values should be interpreted according to the ROS positive and negative controls.

2. Incubate the cells until 50-70% confluence is reached, approximately 10^4 cells/well (times may vary depending on the cell type)[2].

3. Prepare a 1:100 dilution of the non-fluorescent CONTROL SPAchips in cell culture medium[3]. Mix thoroughly (vortex). Do not spin the tubes.

[1]It is highly recommended the previous counting of SPAchips in a Neubauer chamber. Check SPAchip® count using a routine brightfield microscope.

[2]Optimization may be required depending on the cell type and assay conditions. For cells resistant to SPAchips internalization, lipofection may be necessary. Please, contact A4cell for further information.

[3]We recommend to previously test SPAchip® internalization by performing a preliminary experiment using CONTROL SPAchips and checking for % of internalization using a brightfield microscope. This ensures the appropriate quantity and internalization of SPAchips, allowing cell assay optimization.

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4. **Dilute** ASSAY SPAchip® stock solution in cell culture medium to obtain a final SPAchip® to cell ratio[4] of 3:1. Alternatively, simply calculate 1 µL of stock per assay well and dilute 1:100 in cell culture medium. Vortex the ASSAY SPAchip® tube right before using it. Pipette up-and-down twice to homogenize the ASSAY SPAchip® solution and do not spin the tube.

5. If fluorescence baseline is required, CONTROL SPAchip® samples (non-fluorescent) can be included in assay design. In a 96-well plate, aspire the cell medium and add 100 µL of CONTROL SPAchip® **dilution** to the control wells (Figure 1). Vortex the tube right before use. Add 100 µL ASSAY SPAchip®-containing fresh culture medium per well[5]. Homogenize the solution by pipetting up and down several times.

6. **Incubate** overnight in a cell incubator to let the cells to internalize the SPAchip®. Internalization rate may vary for each cell type, regular values for adherent cells range from 25 to 50% depending on confluence, SPAchip®-to-cell ratio and assay duration.

7. Use some wells of the plate for positive and negative ROS controls (see example of plate template below in Figure 1).

8. Perform the experiment with your read-out platform. For long-time kinetic assay (for example, monitorization during one week or month), keep the plate under proper conditions (CO_2, temperature and, humidity) between each measurement and change the medium accordingly, depending on the cell type.

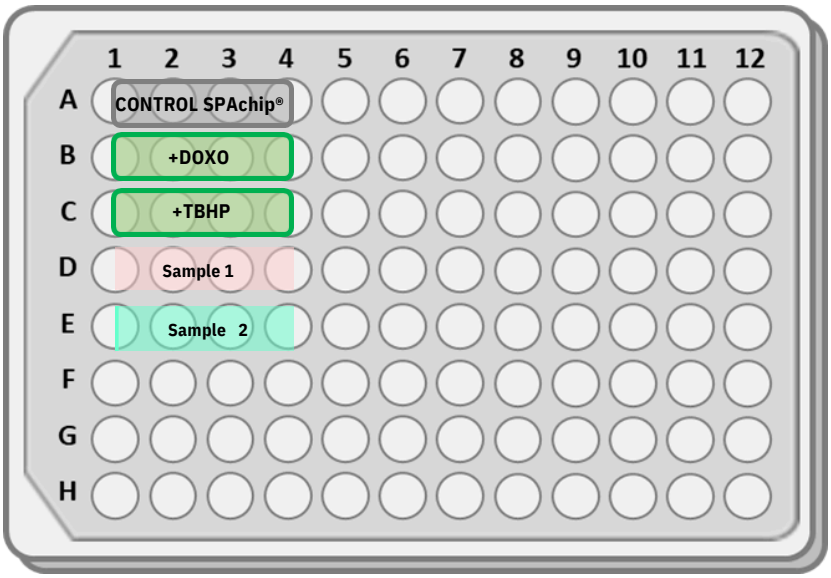

Figure 1: Example of 96-well plate configuration with positive controls and samples.

[4]It is highly recommended to test SPAchip® internalization in the specific cell line. The appropriate SPAchip® to cell ratio might vary depending on the cell type and assay conditions. Thus, we recommend to previously optimize SPAchip® to cell ratio in previous step, using CONTROL SPAchips.

[5]Phenol Red can quench the signal of some visible wavelength fluorescence dyes and cause problems when used during live cell imaging, interfering in the read-out. We recommend to add a fresh media that does not contain Phenol Red in the formulation.

c. SPAchip® Data Collection

Image Acquisition

1. Set objective to 20x magnification or over (enough resolution for 3 µm SPAchip®)

2. Use transmitted light to localize the region of interest (if using fluorescent light sources use long-wavelength light -561 nm and over- in order to avoid photobleaching of the ROS sensitive probe).

3. Use Z-stacks to capture and set the best focus and the Z position of each SPAchip®. Brightfield images or cell masking stains are recommended for intracellular quantification.

4. Adjust the acquisition parameters for SPAchip® fluorescent signals. ROS channel corresponding to Ex 488 nm /Em 520 nm (FITC fluorescence filter or similar). If using positive controls, to avoid pixel saturation, use higher fluorescence intensity conditions corresponding to DOXO or TBPH wells to adjust this value.

5. Launch the experiment and save the images. Do not change settings parameters while acquisition.

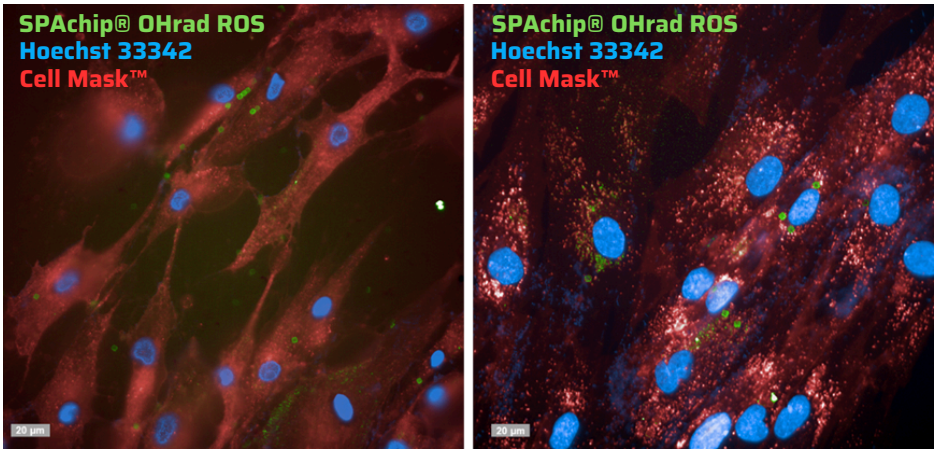

Figure 2: CytoCHECK SPAchip® OHrad ROS Single-Detection Kit in Fibroblast 1095SK cell line.
Representative images showing intracellular ROS SPAchip® in green, acquired with 40x magnification objective. In blue, nuclear staining by Hoechst 33342. In red, cytoplasmatic staining by Cell Mask™ Plasma Membrane. Scale bar: 20 µm.

Flow Cytometry

1. Use FCS and SSC to locate SPAchips and cells independently by their size and complexity.

2. Create fluorescence histograms for green and red channels. Use cells treated with CONTROL SPAchips to set the fluorescence thresholds in both populations (free SPAchips and cells).

3. Analyze a tube of control cells treated with ASSAY SPAchips to verify the fluorescence threshold set in step 2 for gate “Cells”. Positive cells will appear as a separate peak in the histogram.

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d. Quantitative Analysis

1. **Image Analysis:** select SPAchip® fluorescent channel signals (emission at 520 nm for ROS green signal) and measure the intensity of segmented Regions of Interest (ROIs) in every SPAchip® with the image analysis software. Quantify the mean fluorescence of intracellular SPAchip® populations. .
    - For flow cytometry assays: Analyze each sample with the adjusted gating parameters and quantify the channel detection (mean ± SD or median ± rSD).

2. **Quantify** the mean green fluorescence of intracellular SPAchip® populations.

3. **Export** your data to a spreadsheet software.

4. **To determine** the variation of **intracellular reactive oxygen species (ROS)** levels (by specific detection of hydroxyl radicals), use positive and negative controls to obtain relative values to reference control conditions.

5. One of the main advantages of CytoCHECK SPAchip® OHrad ROS Single-Detection Kit is that **OHrad ROS SPAchips are not sensitive to pH changes** (Fig. 3), avoiding measurement artifacts due to pH variations in experimental setup.

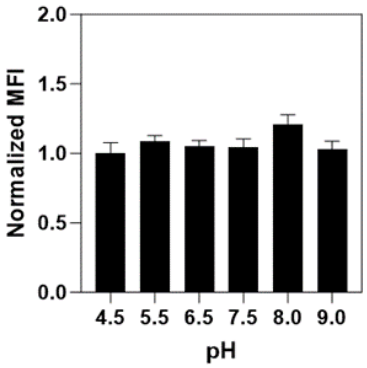

Figure 3: CytoCHECK SPAchip® OHrad ROS Single-Detection Kit is not pH-sensitive.
No significant effect of pH on the fluorescence of CytoCHECK OHrad ROS SPAchips®.

** Contact A4cell staff for assistance with imaging setup and data analysis. Ask for our **cell biology image analysis guide** at **labservices@a4cell.com .***